

RESEARCH PAPER

Transgenic barley lines prove the involvement of *TaCBF14* and *TaCBF15* in the cold acclimation process and in frost tolerance

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Abstract

The enhancement of winter hardiness is one of the most important tasks facing breeders of winter cereals. For this reason, the examination of those regulatory genes involved in the cold acclimation processes is of central importance. The aim of the present work was the functional analysis of two wheat CBF transcription factors, namely *TaCBF14* and *TaCBF15*, shown by previous experiments to play a role in the development of frost tolerance. These genes were isolated from winter wheat and then transformed into spring barley, after which the effect of the transgenes on low temperature stress tolerance was examined. Two different types of frost tests were applied; plants were hardened at low temperature before freezing, or plants were subjected to frost without a hardening period. The analysis showed that *TaCBF14* and *TaCBF15* transgenes improve the frost tolerance to such an extent that the transgenic lines were able to survive freezing temperatures several degrees lower than that which proved lethal for the wild-type spring barley. After freezing, lower ion leakage was measured in transgenic leaves, showing that these plants were less damaged by the frost. Additionally, a higher F_v/F_m parameter was determined, indicating that photosystem II worked more efficiently in the transgenics. Gene expression studies showed that *HvCOR14b*, *HvDHN5*, and *HvDHN8* genes were up-regulated by *TaCBF14* and *TaCBF15*. Beyond that, transgenic lines exhibited moderate retarded development, slower growth, and minor late flowering compared with the wild type, with enhanced transcript level of the gibberellin catabolic *HvGA2ox5* gene.

Key words: Barley, CBFs, frost tolerance, gene expression, gibberellin metabolism, transformation, wheat.

Introduction

Drought and frost are the two most important abiotic stresses which limit crop production in temperate zones. Since winter cereals have to survive freezing temperatures in winter, the examination of cold acclimation and the development of frost tolerance is a prominent research area. Molecular dissection of this complex, multigenic trait

might help to develop more frost-tolerant cereal varieties in the near future. Although cold acclimation is a complex trait and several gene families are involved in this process, the role and the function of the CBF/DREB1 transcription factors have been analysed in the greatest detail in this regard.

Abbreviations: COR, cold-regulated gene; CBF/DREB, CRT/DRE-binding factor; CRT/DRE, C-repeat/dehydration responsive element; FC, fold change; FT, frost test; GP, *Hordeum vulgare* L. cv. 'Golden Promise'.

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The 5' region of several cold or dehydration stress-inducible genes contains a 'CCGAC' core *cis*-motif sequence, the CRT/DRE (C-repeat/dehydration responsive element) (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). CBF transcription factors belong to the CBF/DREB1 subfamily in the ERF family and AP2/ERF (APETALA2/ethylene-responsive factor) superfamily. As their name, CRT/DRE-binding factor (Stockinger et al., 1997; Liu et al., 1998), indicates, they can bind to the promoter of the above-mentioned target genes. CBF proteins are distinguished from the other members of the AP2/ERF protein family by the 'CBF signature' conserved amino acid sequence as well as by the PKK/RPAGRxKFXETRHP and DSAWR motifs present in the N- and C-terminal flanking region, respectively (Jaglo et al., 2001).

CBF genes are expressed transiently in the early phase of the response to low temperature (Stockinger et al., 1997; Gilmour et al., 1998). As the temperature decreases, the transcription of CBF genes becomes more and more intense (Zarka et al., 2003). Chinnusamy et al. (2003) identified *ICE1* in *Arabidopsis* first, and proved that the constitutive overexpression of *ICE1* indeed induces CBF transcription, however only at low temperature, thus enhancing the expression of the CBF regulon and finally increasing frost tolerance. Beside *ICE1*, it has been shown that many other transcription factors and regulatory genes participate in the temperature-related or circadian/diurnal fine tuning of CBF expression in *Arabidopsis*. Recent reviews summarize our current knowledge on this subject (Ruelland et al., 2009; Saibo et al., 2009; Chinnusamy et al., 2010; Thomashow, 2010; Mizoi et al., 2012).

The function of CBF genes has been revealed in many studies in many plant species. In *Arabidopsis*, six CBFs have been identified, and it has been proved that three of them, namely *CBF1/DREB1B*, *CBF2/DREB1C*, and *CBF3/DREB1A*, have a primary role in cold acclimation (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000, 2004; Novillo et al., 2004, 2007). Nevertheless, *CBF4/DREB1D* for example is involved in drought adaptation and it is not induced by low temperature (Haake et al., 2002). Enhanced frost tolerance and induction of *COR* genes were detected in non-acclimated transgenic *Arabidopsis* that overexpressed the *CBF1* gene (Jaglo-Ottosen et al., 1998). Beyond increased frost tolerance, the overexpression of the *CBF3* gene also resulted in improved tolerance to other abiotic (drought, salt) stresses, by the activation of many stress tolerance-related genes even under normal growing conditions in transgenic *Arabidopsis* (Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000). It has also been shown that *CBF2* is a negative regulator of *CBF1* and *CBF3*, suggesting an autoregulation mechanism (Novillo et al., 2004).

In cereals, most information on CBF pathways is available from experiments carried out in barley (*Hordeum vulgare* L.) and wheat (diploid einkorn wheat, *Triticum monococcum*; and bread hexaploid wheat, *Triticum aestivum* L.), and the results from these two species confirm and complement each other. Similarly to *Arabidopsis*, *ICE1*-like genes, namely *TaICE41* and *TaICE87*, were found in wheat as inducers of CBF genes under cold stress (Badawi et al., 2008). Potential downstream

target genes participating in the CBF regulon have been analysed in some studies. CRT motifs or DRE-like elements have been found in the promoter of several cold-regulated or drought-inducible genes in *Triticeae* (reviewed by Cattivelli et al., 2002); among them are *HVA1* (Straub et al., 1994), *DHN5* (Close et al., 1995), *DHN8* (Choi et al., 1999, 2000), and *COR14b* (Crosatti et al., 1996) in barley, and *WCS120* (Vazquez-Tello et al., 1998) in wheat.

Other than in *Arabidopsis*, the CBF genes have been identified and characterized in the greatest detail in cereals. At least 20 and 11 CBF genes were mapped and characterized around the *FR2* (Frost Resistance 2) locus in barley (Skinner et al., 2005, 2006; Tondelli et al., 2006; Francia et al., 2007) and *T. monococcum* (Miller et al., 2006), respectively. To aid comparative analysis, a common gene numbering system was elaborated for the homeologue genes by Skinner et al. (2005) and by Miller et al. (2006). Considering the great numbers of CBF genes encoded in cereal genomes, several experiments have aimed to study the function of individual genes. Skinner et al. (2005) characterized the complex barley CBF gene family using structural, functional, and phylogenetic approaches. Expression analyses in *Triticeae* showed that CBF genes are regulated in a complex way, influenced by genotype, induction temperature, and light-regulated factors (Campoli et al., 2009). Multiplatform candidate gene-based association analysis of 201 rye (*Secale cereale* L.) genotypes showed that single nucleotide polymorphisms (SNPs) in *ScCBF15* and *ScCBF12* genes were significantly associated with frost tolerance (Li et al., 2011). By crossing the frost-tolerant G3116 with the frost-sensitive DV92 diploid wheat genotypes, a mapping population was generated (Miller et al., 2006) and subjected to frost tests (Knox et al., 2008). It was shown that three CBF genes (*TmCBF12*, *TmCBF14*, and *TmCBF15*) were responsible for the improved frost tolerance and this was related to higher expression levels of *COR14b* and *DHN5* genes (Knox et al., 2008). It has been also proved by gene expression studies that three CBF genes, *TaCBF14*, *TaCBF15*, and *TaCBF16*, are induced by cold treatment in hexaploid wheat, and also that their enhanced expression is correlated with the level of frost tolerance (Vágújfalvi et al., 2005). The results of the two aforementioned studies overlap since they highlighted that two CBFs (*CBF14* and *CBF15*) are among those that most effectively participate in the development of frost tolerance. A comparative transcriptome analysis was carried out by Sutton et al. (2009) using two winter wheat genotypes with different levels of frost tolerance. Six wheat CBFs—among them *CBF14*—were found to be differentially expressed in the cold-acclimated winter wheat lines relative to the non-acclimated controls, thus suggesting a possible gain-of-function mutation that leads to an increased level of frost tolerance. These mutations are considered as potential markers for frost survival (Sutton et al., 2009). The association analysis between genetic variants of CBF genes and freezing tolerance revealed that two nucleotide substitutions of *HvCBF14* are statistically associated with freezing tolerance in barley. It was concluded that *HvCBF14* is associated with frost tolerance in a large European barley germplasm collection, suggesting that there is some degree of specificity among the

different *CBFs*, and that *HvCBF14* is the most relevant one for frost tolerance (Fricano *et al.*, 2009). In addition, experiments indicate that the original copy number of a given *CBF* in the genome is also a deciding factor in the level of low temperature tolerance (Knox *et al.*, 2010).

The function of *CBF* genes of temperate cereals has been tested by transformation methods in a few experiments. Overexpression of the wheat *CBF2* gene led to improved frost tolerance and enhanced expression of downstream genes in transgenic tobacco (Takumi *et al.*, 2008). The barley *HvCBF4* gene was overexpressed in transgenic rice and resulted in increased tolerance to low temperature, drought, and high salinity (Oh *et al.*, 2007). In contrast, in a different rice cultivar, the *HvCBF4* transgene caused enhanced survival to drought, but not to high salinity or cold stress (Lourenço *et al.*, 2011).

Besides the enhanced tolerance to abiotic stresses, pleiotropic effects of the constitutively expressed *CBF* transgene were described in several studies. Retarded development, slower growth, and late flowering were observed in different transgenic plant species (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2004; Ito *et al.*, 2006; Lourenço *et al.*, 2011). These effects were eliminated or reduced by using an inducible promoter for the regulation of the transgene (Kasuga *et al.*, 1999; Hsieh *et al.*, 2002; Lee *et al.*, 2003). However several counter-examples show that the constitutive expression of the *CBF* transgene did not result in growth retardation (Jaglo-Ottosen *et al.*, 1998; Oh *et al.*, 2005, 2007; Wang *et al.*, 2008). A likely explanation of the pleiotropic effect of the overexpression of *CBF* in transgenic plants was published by Achard *et al.* (2008). They found that AtCBF1 enhances the accumulation of the growth-repressing DELLA proteins through the alteration of gibberellin (GA) metabolism. DELLA proteins are a small family of growth-repressing proteins, participating in the GA signalling pathway. Bioactive GA synthesis is catalysed by GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox), while the bioactive GAs are inactivated by the GA 2-oxidase enzyme (GA2ox) (for a review, see Yamaguchi, 2008). Accumulation of DELLAs, in parallel with bioactive GA degradation and plant growth retardation, contributes significantly to the cold acclimation process (Achard and Genschik, 2009).

Two *CBF* genes have been detected with prominent roles in the frost tolerance of wheat in earlier studies: *CBF14* and *CBF15* (Vágújfalvi *et al.*, 2005; Knox *et al.*, 2008). In the current work these candidate genes were isolated from winter wheat and overexpressed in spring barley. The effect of the transgenes on the stress adaptation was analysed in response to low and freezing temperatures.

Materials and methods

Plasmid construction

The cDNA of the cold-induced winter wheat (*T. aestivum* ssp. *aestivum*) variety 'Cheyenne' was transcribed by means of reverse transcription (M-MLV RT, Promega) from RNA (TRIzol[®] Reagent, Invitrogen) treated with DNase I (Promega). From the cDNA, *TaCBF14* (accession no. EU076382) and *TaCBF15* (accession no. EU076383) genes were amplified (Accuprime[™] Pfx DNA polymerase, Invitrogen) using a primer pair

including the 4 bp sequences (CACC) necessary for directional cloning on the 5' end of the forward primer (TopoCBF14 forward primer 5'-CACCTAATTACCCCACAGTCG-3', TopoCBF14 reverse primer 5'-TGCTTAGTCGAACAAGTAGCTC-3'; TopoCBF15 forward primer 5'-CACCTAACCAACACTCCTCAG-3', TopoCBF15 reverse primer 5'-AGCTGGCTGGAGTGTCTTAGTA-3'). These fragments were cloned into the pENTR[™]/D-TOPO[®] (Invitrogen) Gateway-based cloning vector, followed by an LR recombination reaction between the cloning (donor) vectors and the pBract214 binary recipient vector (<http://www.bract.org/constructs.htm#barley>), in which the *hpt* selectable marker gene (encodes hygromycin phosphotransferase) confers resistance to the antibiotic hygromycin B, and the maize ubiquitin promoter+intron (Ubi1) ensures the constitutive expression of the transgene (see Supplementary Fig. S1A–D available at JXB online). Validation of the pBract214-*TaCBF14* and pBract214-*TaCBF15* constructs was carried out by means of PCR, digestion with restriction enzymes (*Bam*HI and *Sac*I; Fermentas), and sequencing (BRC, DNA Sequencing Laboratory, Szeged, Hungary); then they were transformed into the *Agrobacterium tumefaciens* strain AGL1.

Plant material and transgenic plant production

Immature embryos of the spring barley (*H. vulgare* L.) cultivar 'Golden Promise' (GP) were transformed by the method of Bartlett *et al.* (2008) and Harwood *et al.* (2009) with *A. tumefaciens* strain AGL1 carrying the binary vector pBract214-*TaCBF14* or pBract214-*TaCBF15*. Transformation was also carried out with the pBract202 vector (carrying only the *hpt* selectable marker gene), producing transgenic control plants.

Molecular characterization of the transgenic barley plants

Plant genomic DNA was extracted from the leaves of the regenerated lines (DNeasy Plant Mini Kit; Qiagen). Transgenic plants were verified by PCR with the above-mentioned TopoCBF primers and with specific primers which can bind to the selectable marker gene *hpt*: HygF 5'-ACTCACCGCAGCTCTGTC-3'; HygR 5'-GCGCGTCTGCTGCTCCAT-3' (Stanley *et al.*, 2011). The forward primer on the ubiquitin promoter is: pAH Ubi_promD primer forward 5'-GCATATGCAGCAGCTATATGTG-3'; and the reverse primer on the NOS terminator is nostermin_3' reverse primer: 5'-GATATCAGCTTGCGATGCCGGTC-3'.

The determination of transgene copy number in the T₀ and T₁ generation was carried out at IDNA Genetics Limited, UK, using quantitative real-time PCR with primers and probes specific for the hygromycin gene. In the T₁ generation, copy number analysis was used to determine whether the transgene was inherited in the homozygous or heterozygous state in the progeny plants.

Frost tests

Frost tests were performed according to Vágújfalvi *et al.* (2003). After 3 weeks of preliminary growth under control conditions (17/13 °C day/night temperature, 16 h illumination, 240 µmol m⁻² s⁻¹ light intensity, 75% relative humidity), the plants were cold hardened for 3 weeks at 4 °C (under the same illumination conditions). In the case of frost testing without cold hardening, plants grown under control conditions were subjected to the frost directly. Hardened plants were frozen at -11 °C and -13 °C, while the non-hardened plants were treated at -6 °C. Twenty individual plants were evaluated for each line in the experiments.

Frost damage to the plants was scored directly on the basis of rate of recovery (scoring). It was evaluated 1 and 2 weeks after frost on a scale ranging from 0 (perished by cold) to 5 (no frost damage). The survival percentage for each line was calculated as the ratio of surviving plants to the number of plants subjected to freezing temperature. Fluorescence induction parameters (F_v/F_m ratio and F_0) were determined in leaves with a pulse amplitude-modulated fluorometer

(PAM 2000, Walz, Effeltrich) to evaluate the increased cold acclimation capability and frost tolerance (Rizza et al., 2001). Conductance measurements were taken on leaf tissue subjected to frost; a 3 cm segment of the green, photosynthetic leaf tissue was used. Samples were taken from 10 plants for each line and put into 15 ml Falcon tubes with 5 ml of MQ water. They were shaken overnight at 300 rpm before recording the conductivity with an electrical conductivity meter (Mikro KKT). Then they were autoclaved for 20 min, and cooled to room temperature before measuring the total potential conductivity. Values were adjusted by subtracting the conductivity of the deionized water. Relative conductivity of frost-treated samples represents the adjusted mean ion leakage as a percentage of the total adjusted leakage after boiling. These parameters were measured at the end of the hardening period, immediately after the freezing temperature, as well as 24 h and 48 h after freezing on 10 plants for each line.

Low temperature stress treatment

Samples for gene expression studies were taken under control conditions (17/13 °C day/night temperature, 16 h illumination, 240 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 75% relative humidity) and after 24 h cold treatment at 4 °C. In both cases, lighting was switched on at 08:00 h, and the samples were collected at 09:30 h (i.e. after 90 min illumination). Samples from five plants were pooled and homogenized.

RNA expression analyses

Total RNA from leaves was extracted with TRIzol® Reagent (Invitrogen) following the manufacturers instructions. 1 μg aliquot of RNA samples was reverse transcribed using M-MLV Reverse Transcriptase (Promega) with oligo(dT)₁₅ primer (Promega). The expression level of the transgene and a number of target genes was determined by means of real-time PCR (ABI 7500 Fast Real-Time PCR instrument, Applied Biosystems) using Brilliant® II SYBR® Green QPCR Master Mix (Stratagene). The relative fold change (FC) values were calculated with the $\Delta\Delta\text{Ct}$ method (Bookout and Mangelsdorf, 2003). The sequences of the quantitative PCR primers are listed in Table 1.

Statistical analysis

The statistical evaluation of the data was performed with the SPSS statistical program package version 16.0. Outliers were eliminated ($\alpha=0.05$). The normality of the data sets was checked with Kolmogorov–Smirnov test and the equality of variances using Levene's test. Pairwise comparison (transgenic lines compared with the wild type) was performed applying the least significant difference (LSD) method when the variances were homogeneous, while the Tamhane test was used to find significant differences between the lines if the variances were unequal. *t*-test was used to compare two measuring points.

Results

For the plant transformation, the coding regions of the candidate *TaCBF14* and *TaCBF15* genes were cloned into pBract214 vector, developed for overexpression in cereals. Immature barley embryos were transformed using constructs pBract214-*TaCBF14* and pBract214-*TaCBF15*. Moreover, a control empty vector, pBract202, carrying only the *hpt* selectable marker gene, was used to produce hygromycin-resistant transgenic control plants.

The transformation efficiency is summarized in Table 2. Only the plants which originated from different calli were considered as independent transgenic lines. The integration of the transgene was demonstrated by PCR (see Supplementary Fig. S2A, B at JXB online); all of the candidate transgenic lines proved to be PCR positive in the T₀ generation. The transgene copy number was determined too; plants with low copy number (2–4) and in most cases with a single transgene copy were produced (see Supplementary Table S1).

In the transformants, the transgene is regulated by the ubiquitin promoter, which ensures strong constitutive expression.

Table 1. The primer pairs (Fwd, forward primer; Rev, reverse primer) of the genes for quantitative RT-PCR.

Gene names	GenBank (NCBI)	Primer pair sequences		Reference of primer pair
<i>HvCyclophilin</i> (reference gene)	AK253120.1	Fwd	5'-CCTGTCGTGTCGTCGGTCTAAA-3'	Burton et al. (2004)
		Rev	5'-ACGCAGATCCAGCAGCCTAAAG-3'	
<i>TaCBF14</i> (transgene)	EU076382	Fwd	5'-AACCAGATGACGAGAAGGAAA-3'	
		Rev	5'-AACTCCGAGTAGCAGCATCC-3'	
<i>TaCBF15</i> (transgene)	EU076383	Fwd	5'-GTCGTCCATGGAAAATACCG-3'	
		Rev	5'-ATGTGTCCAGGTCCATTTC-3'	
<i>HvCBF1</i>	AY785839	Fwd	5'-ATGGACGATGGTATGGACTTC-3'	Morran et al. (2011)
		Rev	5'-TGCACATGCATTAGTAGTTC-3'	
<i>HvCBF6</i>	EU332012	Fwd	5'-TGGGATGGGACCTTTACTACG-3'	Morran et al. (2011)
		Rev	5'-GCATCAATCGGAAGCCAAGAC-3'	
<i>HvCBF9</i>	AY785877	Fwd	5'-AGCACTACTGTCAACATGTAG-3'	Morran et al. (2011)
		Rev	5'-CCTTGATTCGATTCATGGAG-3'	
<i>HvCOR14b</i>	AJ512944	Fwd	5'-TTGAGGATGTGAGCAAATGAG-3'	Morran et al. (2011)
		Rev	5'-TACATCGTCAATGACGAGACC-3'	
<i>HvDHN5</i>	AF043096	Fwd	5'-CCACCAGCATACCACTGAGACC-3'	Campoli et al. (2009)
		Rev	5'-TAGTGCTGTCCAGGCAGCTTGT-3'	
<i>HvDHN8</i>	AF181458	Fwd	5'-TGCTCCAGCGCCAGTGCAC-3'	Campoli et al. (2009)
		Rev	5'-CGATCAAGCTCTGGGCTTGTG-3'	
<i>HvGA2ox4</i>	AY551432	Fwd	5'-TCCTAGCCAGCCAGCAACT-3'	Dewi (2006)
		Rev	5'-GGCATGGACAGGACACAGA-3'	
<i>HvGA2ox5</i>	AY551433	Fwd	5'-ACAAGAGCAGCACCCACAA-3'	Dewi (2006)
		Rev	5'-AACCACAGGACCAGGACGA-3'	

Table 2. Summary of the transformation experiments showing transformation efficiencies, defined as the number of independent transformed plants as a percentage of the number of immature embryos treated (Bartlett *et al.* 2008; Harwood *et al.*, 2009).

Constructs	No. of immature embryos co-cultivated	No. of regenerated green plants	No. of independent transformed lines	Transformation efficiency (%)
pBract202	60	14	2	3.33
pBract214-TaCBF14	137	28	10	7.30
pBract214-TaCBF15	146	47	18	12.33

Samples were thus taken under control conditions to demonstrate the function of the transgene (see [Supplementary Fig. S3A, B](#) at *JXB* online). Accordingly, the expression level of the transgene was shown by quantitative RT-PCR in all the independent lines of the T₀ generation (see [Supplementary Fig. S4A, B](#)).

Development of transformant plants

When T₀ plants were regenerated successively from tissue culture, differences in the phenotype between the transformants and GP could not be discerned. It was observed in the T₁ and T₂ progeny that the majority of the lines overexpressing the *TaCBF14* and *TaCBF15* genes exhibited moderately retarded development (see [Supplementary Fig. S5A, B](#) at *JXB* online), slower growth, and slightly later flowering compared with the wild type under normal plant growth conditions ([Fig. 1](#)). [Table 3](#) shows the time of heading based on the Zadoks scale (Zadoks *et al.*, 1974; Murray and Robertson, 2003). Lines CBF14_L8, CBF15_L1, and CBF15_L6 were the most delayed in their development; they entered into the heading phase 2–3 weeks later than the wild type. The transgenic control line eared at the same time as the wild type, and no difference was observed in the course of their development.

Frost tests on cold-hardened plants

In order to select the most tolerant *TaCBF* transgenic barley lines, all 28 independent transformants and one transgenic control line were tested for frost tolerance and compared with wild-type GP barley. After a 3 week long hardening phase at 4 °C, freezing temperatures of –11 °C and –13 °C were applied in the first frost tests on the T₁ generation. Some of the transformants survived even at the lower freezing temperature. To confirm the results, the frost test at –13 °C was repeated in a second experiment. The most resistant lines were identified on the basis of rate of recovery (scoring), survival percentage, chlorophyll fluorescence measurements, and conductance tests ([Table 4](#)). Based on their overall performance, the best lines were selected for further analyses (indicated by bold type in [Table 4](#)). In the homozygous T₂ generation, these lines were tested at –11 °C and –13 °C. Judging by the conductance measurements, the leaves of the transformants were less severely injured by freezing than those of the wild type (see [Supplementary Table S2](#) at *JXB* online). The F_v/F_m values were significantly higher in the leaves of transgenic plants, indicating

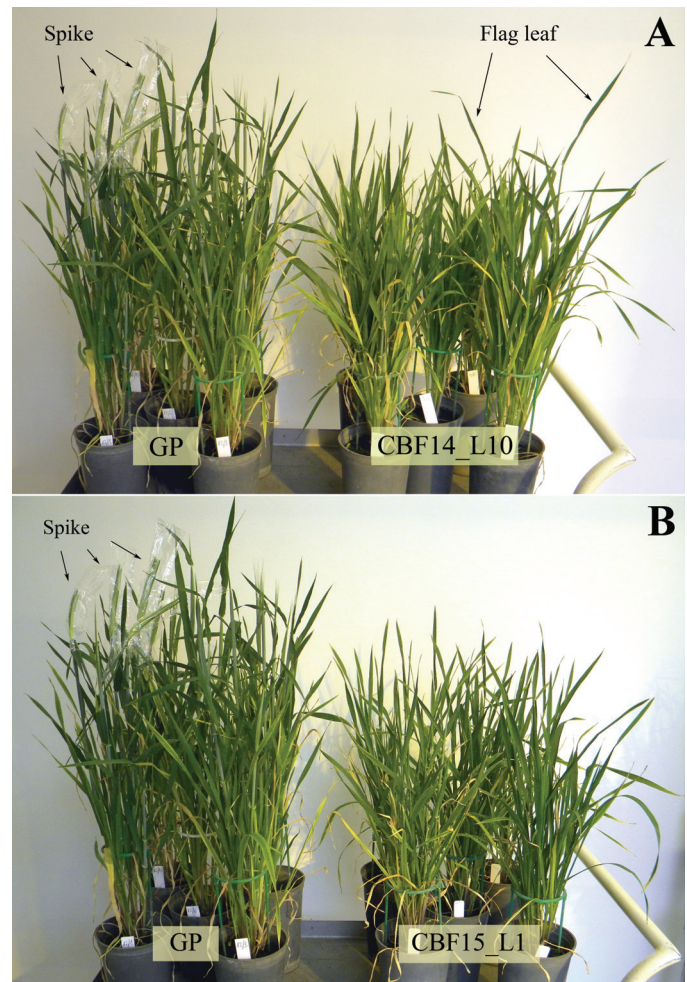


Fig. 1. Transgenic lines [CBF14_L10 (A) and CBF15_L1 (B)] show moderate retarded development, slower growth, and slightly later flowering compared with the wild-type Golden Promise (GP).

less damage to photosystem II (PSII) (see [Supplementary Table S3](#)). The six selected *TaCBF14* transformants and line CBF15_L1 also proved to be more frost resistant than wild-type GP on the basis of survival percentage ([Fig. 2](#)) and rate of recovery (see [Supplementary Fig. S6A, B](#)). The other *TaCBF15* transformants tested exhibited a slight increase in frost tolerance. The results obtained in the homozygous T₂ generation showed bigger differences between the wild type and transgenic lines than those recorded in frost tests on the T₁ generation, and the differences compared with the wild type were more pronounced.

Table 3. The development of the barley lines in the period of flowering (89–14 d after germination) is shown based on the Zadoks scale (Zadoks et al., 1974; Murray and Robertson, 2003).

Days	GP	TC_L1	CBF14_L4	CBF14_L6	CBF14_L8	CBF14_L10	CBF15_L1	CBF15_L6	CBF15_L14
89	47	47	41	47	41	41	39	39	47
105	55	55	45	53	41	41	41	41	53
119	59	59	57	57	53	53	45	45	57
132	73	73	57	57	53	55	53	53	57
145	77	77	59	59	55	57	55	55	59

GP, Golden Promise wild-type barley; TC_L1, transgenic control line; CBF14_L4–L10, TaCBF14 transgenic lines; CBF15_L1–L14, TaCBF15 transgenic lines.
Numbers on the scale indicate: 39, flag leaf collar just visible; 41, flag leaf sheath extending; 45, boots just swollen; 47, flag leaf sheath opening; 53, a quarter of inflorescence emerged; 55, half of inflorescence emerged; 57, three-quarters of inflorescence emerged; 59, emergence of inflorescence completed; 73, early milk; 77, late milk.

Frost test on non-hardened plants

As the *TaCBF14* and *TaCBF15* transgenes are constitutively expressed, frost tests were also performed on the selected lines without a preliminary hardening period. Treatment at –6 °C was lethal for the wild type, while the transgenic lines exhibited a low percentage of regeneration after freezing (Table 5). In this experiment, lines CBF14_L10 and CBF15_L1 proved to have the best frost tolerance.

Expression analysis

The expression levels of some genes belonging to the late embryogenesis abundant (LEA) protein group, and several cold-inducible barley *CBF* genes were tested in control and cold-treated samples of wild-type GP and three selected lines each from TaCBF14 and TaCBF15 transgenics (T₃ progeny), which proved to be most resistant in previous physiological tests. Also the expression level of the transgene was analysed in the samples (Fig. 3). Primer pairs were designed for the *TaCBF* transgenes (Table 1), and were also tested on the wild-type barley samples. *CBF* transcript was not detectable in the control GP samples with these primers. However, after cold treatment, those primers could amplify some transcript with low efficiency. This expression level was orders of magnitude lower than detected in transgenics, so these values are simply not visible on Fig. 3. The expression of the *TaCBF14* or *TaCBF15* transgene was enhanced after cold treatment in the CBF14 lines and in CBF15_L1. However, cold-induced transgene expression changes were not detected in the two further CBF15 transgenics (L6 and L14) examined.

The expression levels of the cold-inducible barley *HvCBF1*, *HvCBF6*, and *HvCBF9* genes were also analysed in the same set of transgenics (Fig. 4). The FC value of the GP control sample was assigned as 1. *HvCBF1* (Fig. 4A) and *HvCBF9* (Fig. 4C) were expressed to the same extent under control conditions in all the lines tested. After cold treatment it was established that these genes were induced strongly in GP. On the other hand, the level of cold induction of these genes was not so high in the majority of the transgenic lines. In the case of *HvCBF6* (Fig. 4B), a similar phenomenon was not observed, except in the line CBF14_L4 where the FC values

of cold-treated transgenic samples were lower than in the wild type. The expression level of *HvCBF6* was less than half in the control samples of CBF14_L4 and CBF15_L6 lines, and only one-fifth of the FC value of the wild-type control sample was measured in CBF14_L10 and in CBF15_L1. Expression analysis of other barley *CBF* genes, namely *HvCBF2A*, *HvCBF3*, *HvCBF10A*, *HvCBF11*, *HvCBF14*, *HvCBF15*, and *HvCBF16* (using the primer pairs reported and the sequences kindly provided by Morran et al., 2011), was carried out on the two best transgenic lines (CBF14_L10 and CBF15_L1) and compared with wild-type GP; however, differences were not detected between the lines (data not shown).

One of the largest groups of genes induced by drought, cold and salt stress consists of the LEA proteins (Ingram and Bartels, 1996; Thomashow, 1999). The relative expression of three genes belonging to the LEA protein group was analysed on the above-mentioned lines (Fig. 5). The *HvCOR14b* gene was induced in the wild type only by cold treatment, while in the transgenic lines its expression was improved in control conditions too and it was enhanced more by cold stress (Fig. 5A). Also the expression of the *HvDHN5* gene was greater (50- to 100-fold) in transgenic lines than in GP in control conditions (Fig. 5B). In CBF14_L10 and CBF15_L1, 10 times greater *HvDHN8* expression was recorded under control conditions than in GP (Fig. 5C). After cold treatment, this gene was more highly expressed in CBF14_L4 and CBF14_L10.

Because of the possible effect of TaCBF14 and TaCBF15 transcription factors on GA metabolism, using the most tolerant transgenic lines and the wild-type GP, the expression of some genes which encode enzymes that have roles in the later steps of the GA biosynthetic pathway was analysed. In GP, *HvGA20ox1* and *HvGA20ox3* genes were induced after cold treatment to a small degree; their expression was 2-fold that measured in control samples. The expression levels of these genes were similar in the transgenic lines (see Supplementary Fig. S7A, B at JXB online). Differences between the lines and GP wild type were not detected in the expression level of *HvGA20ox4* and *HvGA20ox5* genes in control conditions (Fig. 6). After 1 d cold treatment, the transcript levels of the bioactive GA-inactivating *HvGA2ox* genes were enhanced in the samples. The most conspicuous changes were

Table 4. Results of the first and second frost tests (FT). Evaluation is based on the significance level of the measurements (F_v/F_m parameter, rate of recovery, survival percentage, and conductance).

Lines	First FT		Second FT						Σ^*		Survival (%)	
	F_v/F_m		F_v/F_m		Rate of recovery		Conductivity		Rate of recovery		Survival (%)	
	24 h	48 h	24 h	48 h	1 week	2 weeks	24 h	48 h	1 week	2 weeks	First FT	Second FT
	-11 °C	-13 °C	-13 °C	-13 °C	-11 °C	-13 °C	-13 °C	-13 °C	-13 °C	-13 °C	-11 °C	-13 °C
CBF14_L1	**		*	***	*	***	*	***	*		19	31.6
CBF14_L2			*		*		*				5	10
CBF14_L3											5	
CBF14_L4	***	***	***	***	***	*	***	***	***	**	27	15
CBF14_L5			***	***	***	***	***	***	***	***	6	18.8
CBF14_L6		***	***	***	***	***	*	*	*	*	14	22.2
CBF14_L7			***	***	***	*	***	***	*	*	10	10.5
CBF14_L8			*		*	*	*	***	*	*	10	15.8
CBF14_L9			***		*		*		*	*	3	7.69
CBF14_L10	*	***	***	***	***	***	***	***	***	***	21	25
CBF15_L1					*	*	*		*	*	7	11.8
CBF15_L2												10
CBF15_L3			*		*						1	5.88
CBF15_L4												5.56
CBF15_L5			***		***						5	35.7
CBF15_L6					*	*			*	*	3	20
CBF15_L7												9.09
CBF15_L8												5
CBF15_L9												20
CBF15_L10												20
CBF15_L11												20
CBF15_L12												20
CBF15_L13												20
CBF15_L14												20
CBF15_L15												10
CBF15_L16										*		20
CBF15_L17											1	9.09
CBF15_L18												

ND, no data. *, **, *** Significant at the $P \leq 0.05$, 0.01, and 0.001 probability levels, respectively.
The column headed by Σ^* shows the total number of asterisks per line.
Bold type denotes the lines selected for further studies.

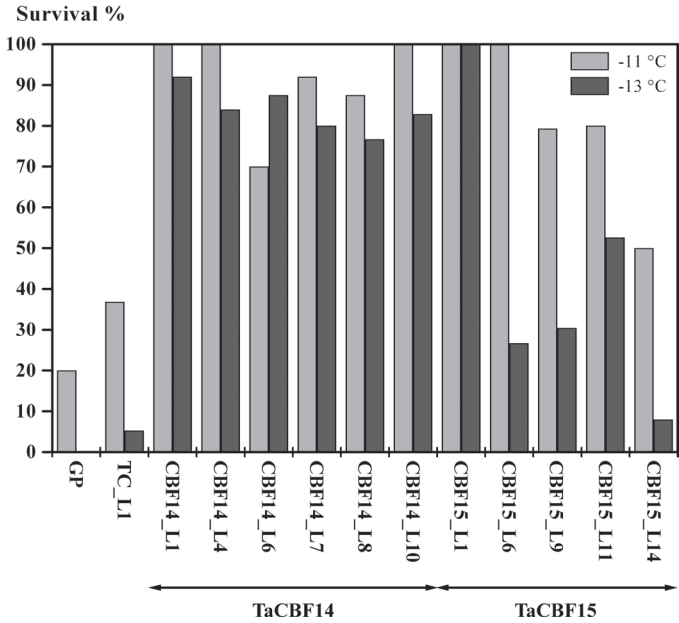


Fig. 2. Survival percentage of the selected TaCBF transgenic barley lines (CBF14_L1–L10 and CBF15_L1–L14) after the third frost test at –11 °C and –13 °C with a hardening period. GP, Golden Promise wild-type barley; TC_L1, transgenic control line.

detected in the expression of the *HvGA2ox5* gene (Fig. 6B) after 1 d cold treatment; this gene was greatly induced in the transgenics compared with the wild type.

Discussion

As mentioned in the Introduction, previous work by the authors and several further studies have highlighted that two CBF transcription factors have an outstanding role in

the development of frost tolerance in wheat. The aim of the current study was to confirm the function of these TaCBF14 and TaCBF15 transcription factors directly by transformation of an economically important cereal. These genes were isolated from winter wheat (*T. aestivum* L. Cheyenne) and the GP spring barley genotype was transformed with them. It was expected that the level of frost tolerance of the recipient plants would be improved by the constitutive overexpression of these candidate genes.

Preliminary test results showed that the wild-type GP spring barley was killed at –10 °C to –11 °C with a 3 week cold hardening phase (Soltész et al., 2012), and at –5 °C without a hardening period (unpublished data) in the frost test system. Because the examined CBF transcription factors were expected to enhance frost tolerance, the transgenic lines were tested at –11 °C and –13 °C after a period of 3 weeks hardening or at –6 °C without hardening. Conductance studies demonstrated that the leaves of transgenics suffered less severe damage from frost than those of the wild type. Significantly higher F_v/F_m values were recorded in the leaves of the transgenics, suggesting that the PSII system was functioning more efficiently in them. Six transgenic TaCBF14 lines and the CBF15_L1 line proved to be more frost resistant than the wild type, and the remainder of the TaCBF15 transgenic lines exhibited a slight increase in frost tolerance. These experiments proved that the TaCBF14 and TaCBF15 transcription factors play an important role in the development of frost tolerance to such an extent that the transgenic lines were able to survive freezing temperatures several degrees lower than that which proved lethal for the wild-type GP. Similar experimental procedures were carried out by Morran et al. (2011) when wheat *TaDREB2* and *TaDREB3* genes—which are related to CBF/DREB1-type transcription factors—were over-expressed in GP spring barley. Three-week-old transgenic

Table 5 Frost test without a cold hardening period

FT (without hardening) at –6 °C	F_v/F_m			Rate of recovery		Survival (%)
	Before the FT	After the FT		After the FT		
		24 h	48 h	1 week	2 weeks	
GP	0.798	0.764	0.771	0.43	0.00	0
TC_L1	0.789**	0.755	0.769	0.10*	0.00	0
CBF14_L1	ND	ND	ND	1.00*	0.25	10.53
CBF14_L4	0.779***	0.765	0.765	0.90	0.35	15.00
CBF14_L6	0.792*	0.771	0.771	0.70	0.40	16.67
CBF14_L7	ND	ND	ND	0.55	0.25	10.53
CBF14_L8	0.792	0.769	0.770	0.51	0.00	0
CBF14_L10	0.786***	0.766	0.767	1.60**	0.69***	26.32
CBF15_L1	0.789**	0.781*	0.783	1.80***	0.69***	15.79
CBF15_L6	0.787***	0.763	0.778	0.88	0.28	11.11
CBF15_L9	ND	ND	ND	0.55	0.28	10.53
CBF15_L11	0.791*	0.762	0.775	0.53	0.13	10.53
CBF15_L14	0.798	0.777	0.780	0.60	0.00	0

GP, Golden Promise wild-type barley; TC_L1, transgenic control line; CBF14_L1–L10, TaCBF14 transgenic lines; CBF15_L1–L14, TaCBF15 transgenic lines; ND, no data.
*, **, *** Significant at the $P \leq 0.05$, 0.01, and 0.001 probability levels, respectively.

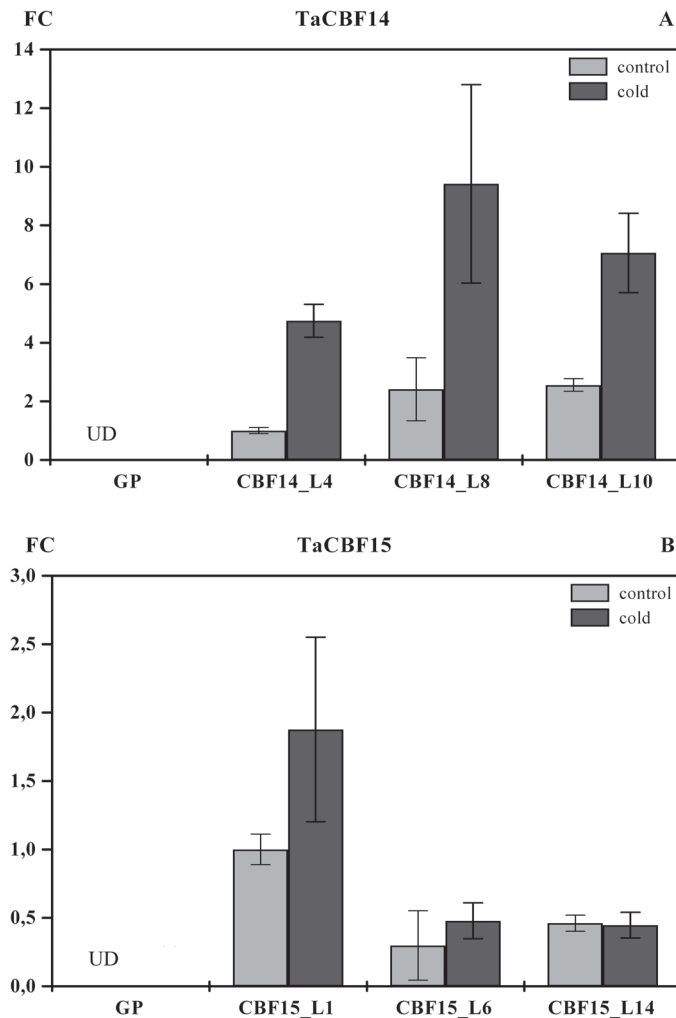


Fig. 3. Relative expression of the *TaCBF14* (A) and *TaCBF15* (B) transgene is shown in control and 1 d cold-treated transgenic barley lines. The fold change (FC) values of control samples CBF14_L4 and CBF15_L1 were assigned as 1. Exogenous barley *CBF* expression was detected in wild-type Golden Promise (GP) after cold; the value was FC=0.000352 in the case of the *TaCBF14* primer pair, and FC=0.000033 in the case of the *TaCBF15* primer pair. UD, undetectable.

plants showed a 25–55% survival compared with the wild type after a short -6°C frost treatment, carried out without a hardening period.

The function of the transgenes was checked under control conditions and after 1 d cold treatment in the selected transgenic lines (T_3 generation) based on frost tests. Cold-induced gene expression enhancement was observed in *TaCBF14* lines and in *TaCBF15_L1*, even though a constitutive—maize ubiquitin (*Ubi1*)—promoter was used to regulate the transgene. It was already reported by Christensen and Quail (1996) that this promoter is both thermal and mechanical stress inducible in transgenic rice. Most probably the *Ubi1* promoter is also cold induced in barley and/or its efficiency may be affected by the transgene integration site. Because of the high homology of the wheat and barley *CBF* genes, the primers designed for transgene expression measurements

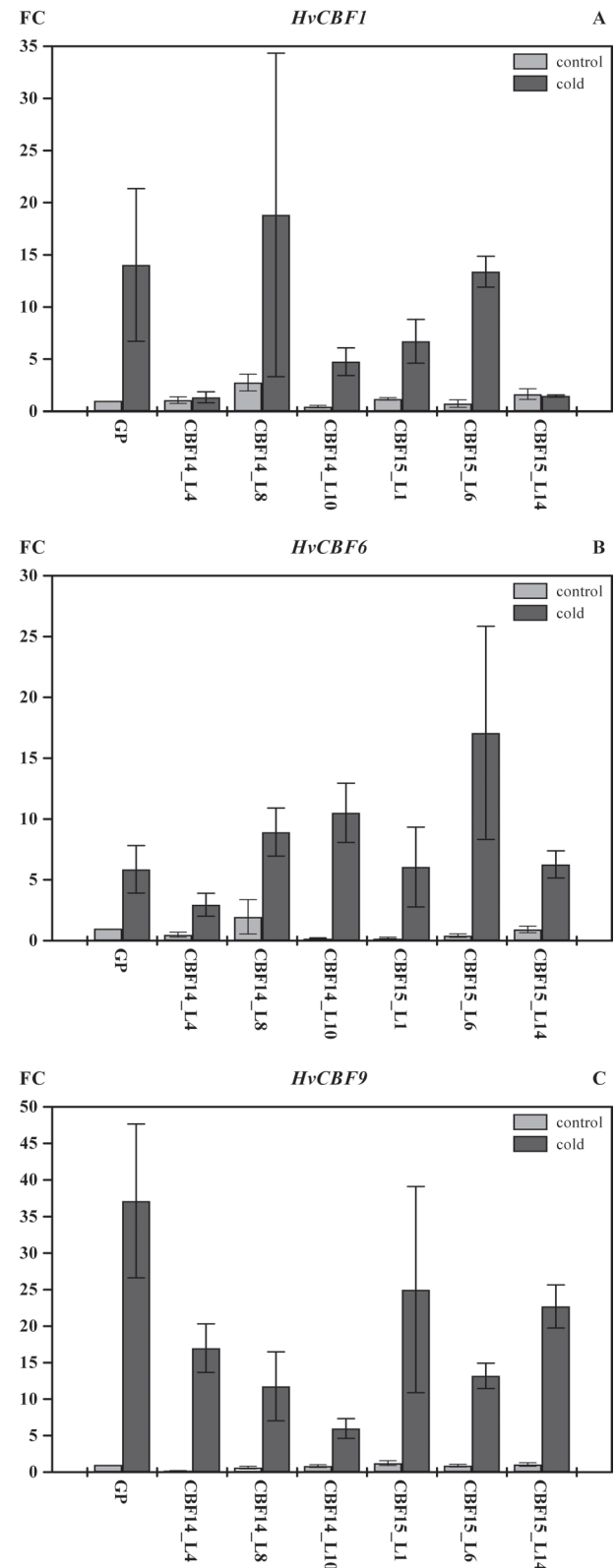


Fig. 4. Expression of *HvCBF1* (A), *HvCBF6* (B), and *HvCBF9* (C) genes is shown in the graph in control and 1 d cold-treated samples of three transgenic (*TaCBF14* and *TaCBF15*) barley lines compared with the wild-type Golden Promise (GP). The fold change (FC) value of the GP control sample was assigned as 1.

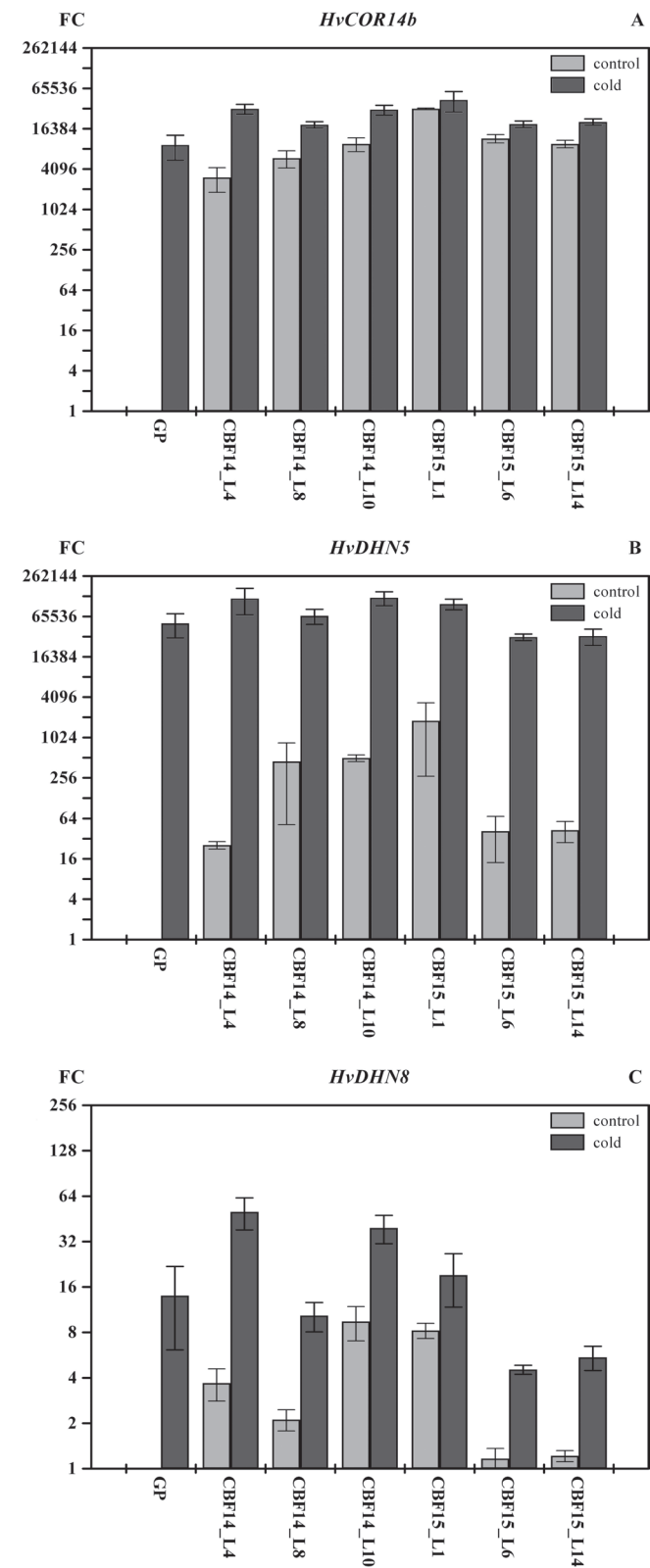


Fig. 5. Expression of *HvCOR14b* (A), *HvDHN5* (B), and *HvDHN8* (C) genes is shown in the graph in control and 1 d cold-treated samples of three transgenic (TaCBF14 and TaCBF15) barley lines compared with the wild-type Golden Promise (GP). The fold change (FC) value of the GP control sample was assigned as 1.

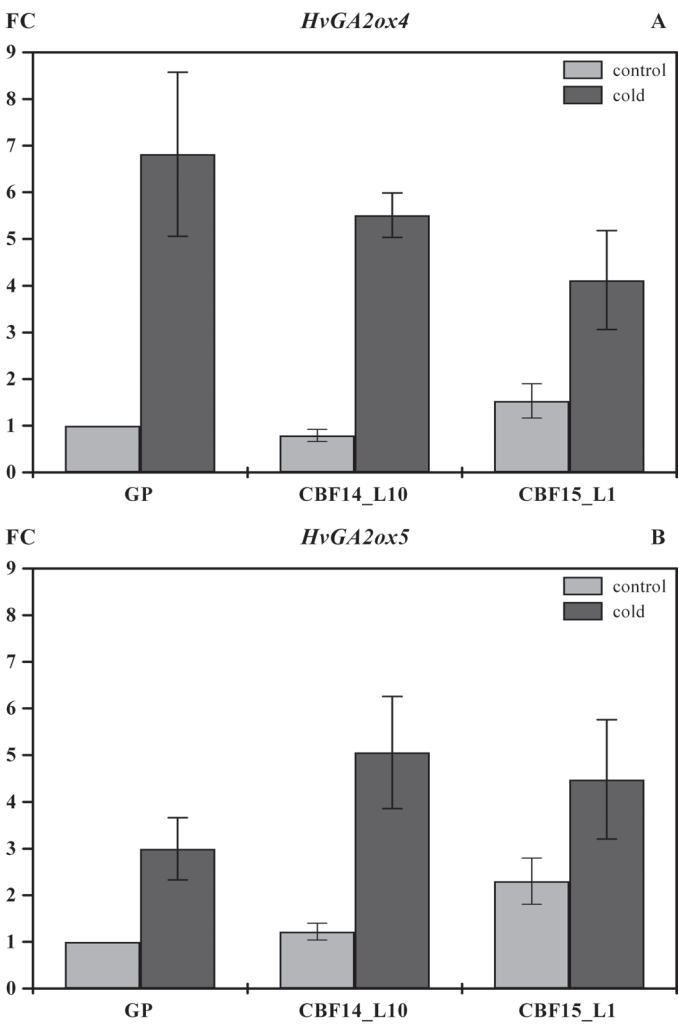


Fig. 6. Expression of *HvGA2ox4* (A) and *HvGA2ox5* (B) genes is shown in the graph in control and 1 d cold-treated samples of the most tolerant transgenic lines compared with the wild-type Golden Promise (GP). The fold change (FC) value of the GP control sample was assigned as 1.

were also tested on the wild-type barley. *CBF* gene expression of the wild-type GP was not detectable under control conditions, although after cold treatment the primers could amplify some transcript at a low level. It is possible that the detected increased transgene expression in the transgenic lines is derived from the co-amplification of the cold-induced exogenous barley *CBF* genes. However, in CBF15_L6 and CBF15_L14, the transgene was expressed constitutively at the same level during the experiment. Because independent transformation events were examined, the genomic background of the integrated copies, the chromatin structure, and nearby enhancer or silencer sequences could be modulating the function of the promoter in independent transgenic lines transformed with the same construct.

The data provided here also show that the ectopic expression of *TaCBF14* and *TaCBF15* influences the function of genes known to play a role in cold hardening or in the development of frost resistance in barley. Changes were detected in the expression of certain genes belonging to the CBF regulon.

The greatest effect was seen for the *HvCOR14b* gene, which is induced normally by low temperature stress (Crosatti *et al.*, 1996, 1999, 2003, 2008; Giorni *et al.*, 1999). The enhanced transcript level of the *COR14b* gene is widely used to prove the effectiveness of cold treatment in cereals (Knox *et al.*, 2008; Dhillon *et al.*, 2010). It was found that the constitutive expression of the *TaCBF14* and *TaCBF15* transgenes induced the transcription of *HvCOR14b* in the transgenic barley lines even under control conditions (i.e. without cold treatment); the expression approached the level found after cold treatment in the wild type. Morran *et al.* (2011) observed similarly intense *HvCOR14b* expression in TaDREB3 GP transformants.

Beside *COR*, many other stress-related genes also play an important role in the cold adaptation process and in the development of frost tolerance (for a review in *Arabidopsis*, see Thomashow, 1999; for wheat, see Winfield *et al.*, 2010). In response to cold, the function of dehydrins in cereals is summarized by Kosová *et al.* (2007). The accumulation of the cryoprotective HvDHN5 (Bravo *et al.*, 2003), a homologue of the wheat cold-regulated WCS120, in barley was found to be associated with the induction of frost tolerance (Bravo *et al.*, 1999), and this protein was shown to be accumulated at higher levels in the frost-tolerant genotype than in the sensitive genotype (Zhu *et al.*, 2000). The present results show that *HvDHN5* is up-regulated under control conditions in the *TaCBF14* and *TaCBF15* transgenic spring barley lines. Another dehydrin gene, namely *HvDHN8*, is a homologue of the wheat *WCOR410* gene (Zhu *et al.*, 2000). The cold induction of *HvDHN8* is slower (detectable only after 4 d at 4 °C) than that of *HvDHN5* (highly expressed after 12 h at 4 °C), and, in addition its expression level is lower (Zhu *et al.*, 2000). In the present experiments, enhanced expression of *HvDHN8* was found under control conditions in some transgenic lines; however, its level was lower than in the case of *HvDHN5*. After 1 d cold treatment *HvDHN8* was induced 10-fold in the wild type, while larger increases in gene expression (40-fold and 50-fold) were detected in two *TaCBF14* transgenic lines.

The frost tests and the gene expression analysis prove that the *TaCBF14* and *TaCBF15* genes investigated influence the development of frost tolerance by regulating the *HvCOR14b*, *HvDHN5*, and *HvDHN8* genes. The data also indicate that the *TaCBF14* transcription factor has a larger effect on the development of frost tolerance in transgenic barley than *TaCBF15*.

In *Arabidopsis* it was demonstrated that AtCBF2 is a negative regulator of AtCBF1 and AtCBF3 (Novillo *et al.*, 2004); on the other hand, AtCBF1 and AtCBF3 cannot regulate AtCBF2 (Novillo *et al.*, 2007). The transgenic plants used here provided an opportunity to study whether the overexpression of a *CBF* transgene has any effect on the host-specific (barley-encoded) *CBF* gene expression. Accordingly, the expression of the cold-inducible barley *HvCBFs* was analysed in the transgenic material. The *HvCBF1* and *HvCBF9* genes were induced to a lesser extent in the *TaCBF14* and *TaCBF15* lines in response to cold treatment than GP. However, compared with the wild type, differences in the cold-induced gene expression for other barley *CBFs* were not detected

in the transgenic lines. These results suggest a possible self-regulation system in the *CBF/DREB* gene family in barley. The identification of *cis*-acting or CRT/DRE elements in the promoter region of *CBF/DREB* genes could predict possible autoregulation sites and mechanisms in the ERF family. To the authors' knowledge, there are few published data available on this subject in the cereals. Sequence analysis suggests that the *HvCBF3* gene may not have an autoregulation role (Choi *et al.*, 2002).

As experienced in *CBF* transformation experiments with different plant species (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2004; Ito *et al.*, 2006; Oh *et al.*, 2007), some of the barley transgenic plants, overexpressing the *TaCBF14* and *TaCBF15* transcription factors, also showed retarded development, a slower growth rate, and delayed flowering. Achard *et al.* (2008) found the accumulation of DELLA proteins in lines with increased expression of GA-inactivating *GA2ox* genes in transgenic plants that constitutively overexpressed the *Arabidopsis CBF1/DREB1b* gene. DELLA accumulation was associated with enhanced frost tolerance (Achard *et al.*, 2008). In the present experiments, enhanced transcript levels of the *HvGA2ox5* gene were detected in cold-treated *TaCBF14* and *TaCBF15* transgenic barley lines. Probably the overproduction of the *TaCBF14* and *TaCBF15* genes causes the inactivation of bioactive GAs and the accumulation of DELLA protein. Experiments at the protein level are necessary to elucidate fully the processes in cereals.

The new germplasm developed in the present work was selected after repeated frost tests. These transgenic lines are considered valuable materials for further molecular analysis. Currently, the crossing of the best *TaCBF14* and *TaCBF15* lines is under way. The transcriptome changes caused by the *TaCBF14* and *TaCBF15* transgenes will also be clarified by cDNA microarray analysis. These lines could be used in future experiments aimed at determining the function of the *CBF14* versus *CBF15* regulon.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Maps of the constructions.

Figure S2. Gel photos of the validation of the presence of the transgene in the independent transgenic barley lines.

Figure S3. Gel photos of the validation of transgene expression in the independent transgenic barley lines.

Figure S4. Expression level of the *TaCBF14* or *TaCBF15* transgene in the independent T₀ lines.

Figure S5. Plant height and thousand grain weight data of the transgenic lines in normal plant growth conditions.

Figure S6. Rate of recovery of the transgenic lines after the frost.

Figure S7. Expression of *HvGA20ox1* and *HvGA20ox3* genes.

Table S1. Copy number of the independent transgenic lines.

Table S2. Results of the ion leakage measurement of the third freezing test.

Table S3. Results of the F_v/F_m measurement of the third freezing test.

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